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RESEARCH ARTICLE

Genetic Variation of At-Hook Transcription Factor (Akna) Rs3748178 is Associated with the Relapse of Graves' Disease

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Abstract

Objective: As many as 30-60% of patients with Graves' disease (GD) have relapsed after antithyroid withdrawal, which may be caused by genetic variation of susceptibility factors. This study aimed to analyze the genetic variation of *AT-hook* transcription factor AKNA rs3748178 and its association with relapse in Graves' disease patients. Methods: This research was a case-control study examining 60 DNA samples of GD patients, who were equally allocated into relapse and remission groups. Genetic variation was analyzed with PCR-RFLP. Fisher's exact test was used for statistical analysis and a p-value less than 0.05 was considered significant. Results: The proportion of patients who experienced a relapse with homozygote wild type (n= 27, 90%) was significantly higher (p= 0.015) compared to the heterozygote and homozygote mutant (n=3, 10%). Graves' disease patients with GG genotype were more likely to have a relapse (OR 6.00, 95% CI 1.48–24.3, p= 0.015). The proportion of patients who experienced a relapse with G allotype (95%) was significantly higher (p= 0.004) compared to A allotype (5%). Graves' disease patients with G allotype were more likely to have a relapse (OR 6.33, 95% CI 1.73–23.2, p= 0.004). Conclusions: Genetic variation of *AT-hook* transcription factor AKNA rs3748178 is associated with relapse in GD patients.

Keywords: *Graves' disease, Recurrence, Genetic variation.*

Introduction

Graves' disease (GD) is an autoimmune thyroid disorder characterized by hyperthyroidism, which is an increase in thyroid hormones due to the failure of the body's normal mechanisms of self-tolerance that is causing autoimmune reactions. This fact occurs because thyrotropin receptor (TSHR) is recognized as one of the antigens that results in the formation of autoantibodies [1].

GD management in Indonesia is generally preceded by administration of antithyroid drugs [2]. Patients are declared with GD remission when levels of FT4, FT3, and TSH in serum has reached normal values without medication [3].Antithyroid drug administration is an option that is first performed as the easiest to implement, but it takes 1-2 years to achieve remission. However, the hyperthyroidism recurred in 30-60% of GD patients who discontinued the antithyroid medication [4]. This fact shows that treatment with antithyroid drugs allows high recurrence in patients with GD. Graves' disease is a multifactorial disease [5]. Genetic factors contribute 79% in the pathogenesis of GD [6].

Review of the literature revealed that enlarged thyroid gland, severe Graves' ophthalmopathy, genetic polymorphism of costimulatory genes, low number of regulatory T cells and high TRAb titers might be used to predict the course of disease after the withdrawal of antithyroid drugs [2, 4].Activated CD4 T cells express CD40 ligand (CD154), which interacts with CD40 on B cell

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surface, CD40/CD154 interaction plays an important role in the regulation of autoimmune humoral response.

Studies reported that elevated levels of CD40 and CD154 played a role in the pathogenesis of GD and was associated with the risk of GD recurrence [7,8].CD40 and CD154 expression were regulated tightly at the transcription stage [9]. Regulatory elements of the promoters of CD40 and CD154 gene have the A/T-rich sequence that can be recognized by the transcription factor AKNA (*AT-Hook Transcription Factor*) [10]. This protein has an AT hook-DNA binding motive, which regulates transcription by altering the DNA architecture.

Thus, improving the accessibility of transcription factors to the promoter [11].Genetic variation due to Single Nucleotide Polymorphisms (SNP) of AKNA gene rs3748178 has a distinguished biological role. The single mutation can cause reduction in DNA binding activity to the transcription factor. An elevated level of CD40 and CD154 is associated with the risk of GD relaps [7, 8] and this elevation is regulated tightly in transcriptional stage [9].

It is still unknown whether genetic variation of AKNA, as a transcription factor of both CD40 and CD154, correlates with the risk of GD recurrence. This study aimed to analyze the genetic variation of AT-hook transcription factor AKNA rs3748178 that was associated with relapse of patients with Graves' disease. The results of this study are expected to contribute to development of a screening method in the management of GD.

Methods

This research was a case-control study that was approved by the Research Ethics Committee, Faculty of Medicine, University of Indonesia No.490 / UN2.F1 / ETIK / 2015 and No.204 / UN2.F1 / ETIK / 2016. Laboratory examinations were conducted in 2015-2016 at The Molecular Biology Laboratory of the Medical Biology Department, Faculty of Medicine, Universitas Indonesia.

Subjects

Participants were GD patients who came to the Endocrinology and Metabolism Clinic, Department of Internal Medicine dr. Cipto Mangunkusumo Central Referral Hospital Jakarta, in August-December 2014. The patients were divided into case and control group based on the inclusion and exclusion criteria, then genomic DNA was obtained [2].The case group consisted of GD patients who had been declared remission after receiving antithyroid treatment for at least 12 months, but relapse after 6 months of antithyroid cessation. Inclusion criteria were hyperthyroidism, increased FT4 levels and decreased TSHs and were willing to participate in the study by signing informed consent.

The control group consisted GD patients who had experienced remission after receiving antithyroid treatment for at least 12 months and did not relapse even though the antithyroid drug had been stopped. Inclusion criteria were euthyroid, normal FT4 levels (12.0-27.5 pmol/L) and normal TSH (0.25 - 4.0 mU/L) and were willing to participate in the study by signing informed consent. The exclusion criteria for both groups were radioactive iodine therapy and thyroidectomy.

Sample size was calculated using proportion formula [12]. The formula provides the sample size needed with a critical value of 1.96, proportion of relapse 50% and relative precision of 20%. Based on this calculation, a minimal sample size of 24 subjects was needed for each group. Thirty stored DNA samples from each group were selected randomly for this study, which already exceeded the minimal sample size, thus the power of the study was adequate.

Amplification of AKNA exon 11 using PCR Method

This examination was carried out in the Department of Medical Biology Faculty of Medicine Universitas Indonesia. Stored DNA samples from GD patients who had previously been isolated from 3mL of venous blood using Promega kit DNA purification wizard (ref A1125) were used. Procedures were done according to the instructions in the kit. DNA concentration and purity was calculated using nanodrop (Maestro), with a purity range of 1.8-2.0. Sixty samples of genomic DNA were amplified by PCR technique.

A pair of specific primers for this process was designed based on sequences that were taken from the NCBI data center

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(http://www.ncbi.nlm.nih.gov/) for AKNA gene exon 11 rs3748178, then designed using Primer Quest software from Integrated DNA Technologies (https://sg.idtdna.com/). Primers' specificity was confirmed by the Basic Local Alignment Tool (BLAST). The forward primer (5-'TCC-TCA-TGC-CTC-TGT-TCT-TTG-3') and reverse primer (5'-CGC-CCG-GCC-TAT-TCT-TTA-TTA-3') would produce an amplicon size of 438 bp.

The PCR mixture total reaction volume was 25 µl, and contained 12.5 µl PCR master mix (Kapa Biosystem), 0.25 µl forward primer, 0.25 µl reverse primer, 9.5 µl ddH_2O and 2.5 µl genomic DNA. Amplification was carried out in a PCR Thermal Cycler T100 with PCR condition of 94°C for 5 minutes continued by 35 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 30 seconds and final extension of 72°C for 5 minutes. The PCR products were determined electrophoretically in 1.6% agarose gel.

Determination of Genetic Variation of AKNA Gene rs3748178 using RFLP Method

Ten µl of each PCR product was mixed with 0.5 µl EagI enzyme, 2 µl buffer solution and 7.5 µl ddH_2O. The samples were heated at 37°C for 4 hours, and then loaded onto a 2.4% agarose gel and run in electrophoresis at 80 V for 1 hour. The genetic variation of AKNA gene rs3748178 was determined by comparing the detected PCR fragments to standard fragments that were run in parallel to the samples, which served as a molecular size marker. Homozygous wild type (GG) represented two fragments of 264 bp and 174 bp. Homozygous mutant (AA) represented a fragment of 438 bp. Heterozygote represented three fragments of 438 bp, 264 bp and 174 bp (Fig. 1 and Fig. 2). Three samples were subjected for sequence analysis to confirm the validity of the measurement results.

Statistical Analysis

Statistical analysis was performed using SPSS 22 for Windows. All variables were checked for normal distribution using Kolmogorov-Smirnov test. Fisher's exact test used to compare the genotype and allotype variation between the two groups. A probability of $p < 0.05$ was considered to be statistically significant.

Results

A total of 30 DNA samples of GD relapse patients and 30 DNA samples of GD remission patients were analyzed (Fig. 1 and Fig. 2).

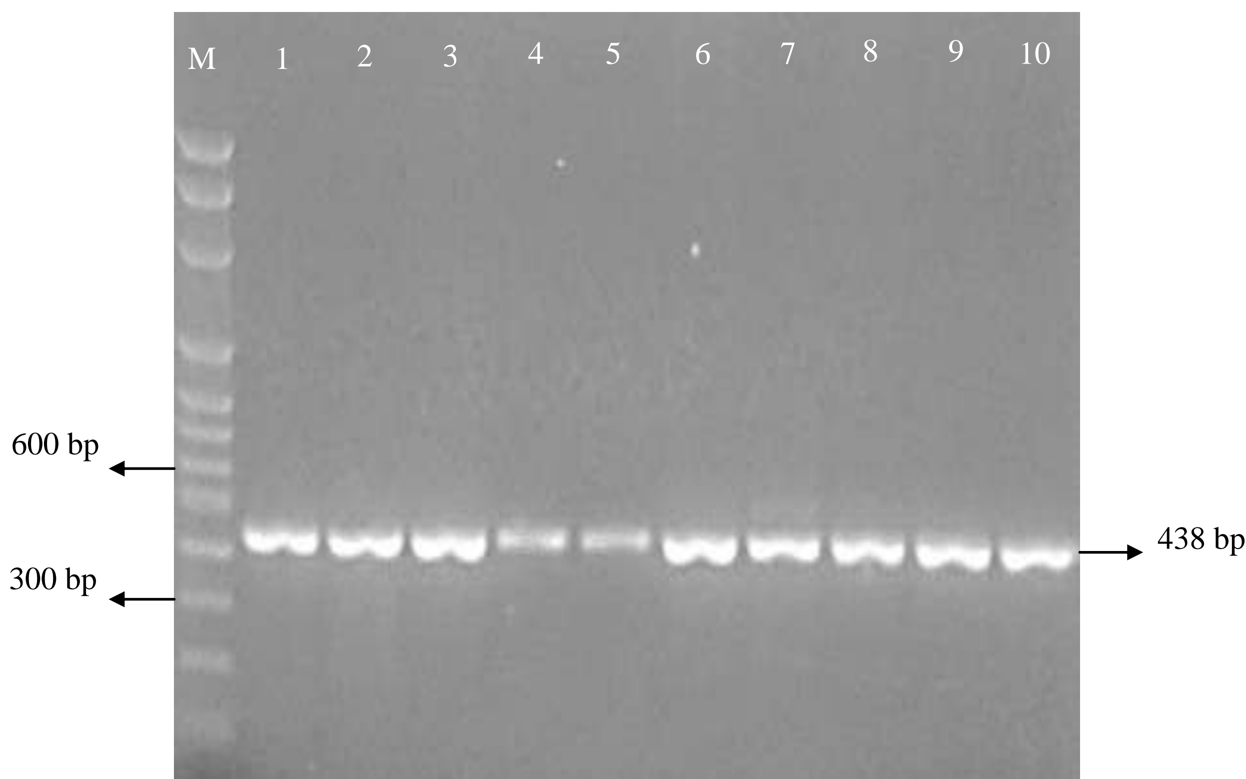

Fig. 1: Fragments of AKNA gene rs3748178 were amplified by PCR, electrophoresed in 1.6% agarose gel and stained with ethidium bromide. The specific amplification characterized by the appearance of a single band with a size of 438 bp. M = marker. Well 1-5 = DNA samples from patients with relapse. Well 6-10 = DNA samples from patients with remission

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Fig. 2: Amplicons of AKNA gene rs3748178 were cut with restriction enzyme EagI, electrophoresed in 2.4% agarose gel and stained with ethidium bromide. Homozygous wildtype (GG) represented two fragments of 264 bp and 174 bp. homozygous mutants (AA) represented a fragment of 438 bp. Heterozygote represented three fragments of 438 bp, 264 bp and 174 bp. The specific amplification characterized by the appearance of a single band with a size of 438 bp. M = marker. Well 1-5 = DNA samples from patients with remission. Well 6-10 = DNA samples from patients with relapse. M = marker. Well 1, 3, 5-9 = GG. Well 2 and 4 = GA. Well 10 = AA

The results showed that there were differences between the genotype GG with GA and AA of AKNA gene rs3748178 in the relapse compared to the remission group (p= 0.015). The proportion of patients who experienced a relapse with GG genotype was significantly higher compared to GA and AA genotype. Graves' disease patients with genotype GG were six times more likely to have a relapse (Table 1).

Table 1: Comparison of AKNA rs3748178 genotype variation between the relapse and remission group of GD patients

		Patient with relapse n (%)	**Patient with remission n (%)**	**OR (CI 95%)**	**p***
Genotype	GG	27 (90)	18 (60)	6.000	0.015
	GA and AA	3 (10)	12 (40)	(1.482-24.299)	
	Total	30 (100)	30 (100)		

*Fisher test
GD= Graves Disease, OR= Odds Ratio, CI= Confidence Interval

There was a difference between the allotype G and A of AKNA gene rs3748178 in the relapse group compared to the remission group (p= 0.004). The proportion of patients who experienced a relapse with G allotype was significantly higher compared to A allotype. Graves' disease patients with allotype G were six times more likely to have a relapse (Table 2).

Table 2: Comparison of AKNA rs3748178 allotype variation between the relapse and remission group of GD patients

		Patient with relapse n (%)	**Patient with remission n (%)**	**OR (CI 95%)**	**p***
Alotype	G	57 (95)	45 (75)	6.333	0.004
	A	3 (5)	15 (25)	(1.726-23.233)	
	Total	30 (100)	30 (100)		

*Fisher test
GD= Graves Disease, OR= Odds Ratio, CI= Confidence Interval

Discussions

Our study showed that genetic variation of AKNA rs3748178 could act as a protective SNP, which the mutant genotype (AA) had a lower recurrence risk of GD. Graves' disease patients with homozygote wild type (GG) were six times more likely to have a relapse. The proportion of patients who experienced a relapse with G allotype was significantly higher compared to A allotype. Graves' disease patients with alotype G were six times more likely to have a relapse. This study focused on genetic variation of costimulatory molecules, which was involved

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indirectly to initiate autoimmune response to thyroid antigens.

Costimulatory molecules are required to generate cytokines and cell surface molecules following initial binding of T-cell receptor to the antigenic peptide-MHC complex. Costimulatory genes play more crucial role than the susceptibility genes in Graves' disease relapse mechanism [4].CD40 and its ligand CD154 are costimulatory molecules, which interact and play an important role in the regulation of autoimmune humoral response. AKNA is their transcription factor, which regulates the transcription of the receptor and its ligand tightly [9, 10].

AKNA has an AT hook-DNA binding motif, which regulates transcription by altering the DNA architecture, thus improving the accessibility of transcription factors to the promoter [11].CD154-CD40 interaction plays a role in the process of differentiation and induction of B cells to produce antibodies to certain antigens. In Graves' disease, B cells produce autoantibodies to TSH receptors that are known as Thyrotropin Receptor Antibodies (TRAb) which have two isoforms, Thyroid Stimulating Antibodies (TSAb) and Thyroid-stimulation Blocking Antibodies (TBAb) [13]. In this study, increased TRAb level was not measured, but several studies proved its role as a risk factor for GD recurrence.

Eliana (2017) confirmed that there was an increase in TRAb levels in serum of recurring GD patients [2]. A significant correlation between TSAb and the severity of ophthalmopathy in GD patients was also confirmed, where this condition was more common in patients who experienced a relapse [14].

AKNA gene encodes a transcription factor that regulates transcription of CD40 and CD154 by AT hook-regulator element binding of both genes. One of the single nucleotide polymorphisms of rs3748178 is located on AKNA gene exon 11, with a variety of bases G/A at nucleotide 114189600 [15].CGG nucleotides that change into CAG cause the transformation of amino acid arginine (R) to glutamine (Q) at codon 1119. The change of R/Q occurs in AT hook-DNA binding motif, precisely at the core of tripeptide GRP (codons 1118-1120) that changes GRP into GQP, which has a less positive charge and reduced DNA binding affinity [16,17]. This change causes a reduced number of CD40 and CD154 protein production, which perhaps lower the risk of GD relapse, since elevated levels of CD40 and CD154 play a role in the pathogenesis of GD and associated with the risk of GD recurrence [7, 8].

Wang demonstrated that rs231775 (OR 1.96, 95% CI 1.18–3.26) at CTLA-4 and rs745307 (OR 7.97, 95% CI 1.01–62.7), rs11569309 (OR 8.09, 95% CI 1.03–63.7), and rs3765457 (OR 2.60, 95% CI 1.08–6.28) at CD40 contribute to the recurrence of GD. Combining risk alleles at CTLA-4 and CD40 might improve the predictability of relapse [4]. This study has some limitations. Even though several studies confirmed that increased TRAb level has a role in GD recurrence, it was not measured due to funding limitation.

To find out whether rs3748178 of AKNA gene was a risk factor for the recurrence of GD in the Indonesian population, a group of non-GD patients are necessary to be added. Further research analyzing all candidates of costimulatory genes that might be involved in GD pathogenesis that are related to AKNA transcription factor (e.g. CD40, CD154) and the interactions between them need to be conducted. These genes and their interactions may have their own impact on outcomes.

Conclusions

Genetic variations of AT-hook transcription factor AKNA gene rs3748178 contributed to the recurrence of GD.

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References

1. Tuncel M (2017) Thyroid stimulating hormone receptor. Mol. Imaging Radionucl. Ther., 9: 26(1):87-91.
2. Eliana F, Suwondo P, Asmarinah A, Harahap A, Djauzi S, Prihartono J, et al (2017) The role of Cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4) gene, Thyroid Stimulating Hormone Receptor

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(TSHR) gene and regulatory T-cells as risk factors for relapse in patients with Graves disease. Acta Med. Indones., 49(3):195-204.

3. Aggarwal R, Chugh P, Basu M (2014) Managing paediatric Graves' disease. Int J Res Med Sci., 2(2):387-91.
4. Wang PW, Chen IY, Juo SHH, His E, Liu RT, Hsieh CJ (2012) Genotype and phenotype predictors of relapse of Graves' disease after Antithyroid Drug Withdrawal. Eur. Thyroid J., 1: 251-8.
5. Girgis CM, Bernard LC, Wall J (2011) Current concepts in Graves' disease. Ther Adv. Endocrinol. Metab., 2(3):135-44.
6. Tomer Y, Huber A (2009) The etiology of autoimmune thyroid disease: a story of genes and environment. J Autoimmun., 32(3-4):231-9.
7. Yamamoto K, Itoh M, Okamura T, Kimura M, Yokoyama A, Yoshino Y, et al (2012) Relative levels of the inflammatory cytokine TNFα and the soluble CD40 ligand profile in serum correlate with the thyrotoxic activity of Graves' disease. Thyroid, 22(5):516-21.
8. Qi Y, Li X, Ma X, Xu L, Zhang X, Jiang X, et al (2014) The role of osteopontin in the induction of the CD40 ligand in Graves' disease. Clin Endocrinol (Oxf)., 80(1):128-34.
9. Cron RQ (2003) CD154 transcriptional regulation in primary human CD4 T cells. Immunol. Res., 27(2-3):185-202.
10. Moliterno AR, Resar LM (2011) AKNA: another AT-hook transcription factor "hooking-up" with inflammation. Cell Res., 21(11):1528-30.
11. Perales G, Burguete-García AI, Dimas J, Bahena-Roman M, Bermudez-Morales V, Moreno H, et al (2010) A polymorphism in the AT-hook motif of the transcriptional regulator AKNA is a risk factor for cervical cancer. Biomarkers, 15(5):470-4.
12. Lemeshow S, Hosmer DW, Klar J, Lwanga SK (1990) Adequacy of sample size in health studies. World Health Organization.
13. Shan SJC, Douglas RS (2014) The pathophysiology of thyroid eye disease. J. Neuro. Ophthalmol., 34:177-85.
14. Subekti I, Boedisantoso A, Moeloek NDF, Waspadji S, Mansyur M (2012) Association of TSH receptor antibody, thyroid stimulating antibody, and thyroid blocking antibody with clinical activity score and degree of severity of Graves ophthalmopathy. Acta Med. Indones., 44(2):114-21.
15. Ota T, Suzuki Y, Nishikawa T, Otsuki T, Sugiyama T, Irie R, et al (2004) Complete sequencing and characterization of 21,243 full length human cDNAs. Nat. Genet., 36(1):40-5.
16. Huth JR, Bewley CA, Nissen MS, Evans JNS, Reeves R, Gronenborn AM, et al (1997) The solution structure of an HMG-I(Y)-DNA complex defines a new architectural minor groove binding motif. Nat. Struct. Biol., 4(8):657-65.
17. Aravind L, Landsman D (1998) AT-hook motifs identified in a wide variety of DNA-binding proteins. Nucleic Acids Res., 26(19):4413-21.

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